Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Hellebrigenol Induces Triple-Negative Breast Cancer Cell Death through ATP1A1 Inhibition

Juan Li
Zunyi Medical University
Xiaorong Huang
Zunyi Medical University
Ying Xu
The Second Affiliated Hospital of Zunyi Medical University
Shuangshuang Fang
The Third Affiliated Hospital of Zunyi Medical University
Liyao Yang
The Third Affiliated Hospital of Zunyi Medical University
Yu Wang
Zunyi Medical University
Danli Yang
Zunyi Medical University
Liwen Shen
shenliwen@zmu.edu.cn
Zunyi Medical University

Research Article

Keywords: Hellebrigenol, TNBC, ATP1A1, Ion homeostasis

Posted Date: October 26th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10833546/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Triple-negative breast cancer (TNBC) represents a highly aggressive malignancy with poor prognosis and limited therapeutic options. The present study aimed to assess the anti-TNBC potential of hellebrigenol, a natural steroid isolated from *Helleborus niger*, and to elucidate whether its cytotoxic effects are mediated by ATP1A1 inhibition. The anti-TNBC activity of hellebrigenol was evaluated by CCK-8 assay, colony formation assay, and flow cytometric analysis. Biochemical assays were employed to measure Na^+/K^+-ATPase inhibition. Cellular thermal shift assays coupled with molecular docking were used to analyze the specific interaction with ATP1A1. The functional relevance of this interaction was validated by genetic rescue with the ATP1A1(Q111R) mutant in conjunction with CCK-8 assay. The role of ion dyshomeostasis in hellebrigenol-mediated cytotoxicity was interrogated by modulating extracellular sodium and potassium levels combined with CCK-8 assay. We found that hellebrigenol significantly suppressed TNBC cell viability and clonogenic capacity, while triggering cell death. Mechanistically, hellebrigenol targeted ATP1A1 and inhibited Na^+/K^+-ATPase activity. Gln111 within ATP1A1 was identified as a critical residue for hellebrigenol binding. Ectopic expression of the ATP1A1(Q111R) mutant completely abolished the compound's cytotoxicity against TNBC cells. Furthermore, exogenous sodium supplementation potentiated hellebrigenol-induced cell death, whereas sodium depletion or potassium supplementation partially reversed its cytotoxic effects against TNBC cells. In conclusion, Hellebrigenol induces TNBC cell death by disrupting ion homeostasis via ATP1A1 inhibition.

1. Introduction

Breast cancer is the most common malignancy and the leading cause of cancer-associated mortality in women [1]. Epidemiological data indicate that there were approximately 2.26 million new cases and 680,000 deaths attributable to this disease worldwide in 2020 [1]. Triple-negative breast cancer (TNBC) is a subtype of breast cancer that lacks estrogen receptor, progesterone receptor, and HER2 expression [2]. The absence of these targets renders TNBC insensitive to the corresponding targeted therapies. At present, clinical management for TNBC depends primarily on surgical resection, radiotherapy, and chemotherapy [3]. Although several novel therapies, such as PARP inhibitors, immune checkpoint blockers, and antibody-drug conjugates, have shown therapeutic benefit in specific patient cohorts, TNBC remains the subtype with the poorest prognosis among all breast cancers, thus underscoring the urgent necessity to develop novel therapeutic strategies [3].

Na^+/K^+-ATPase is a P-type ATPase that extrudes sodium ions (Na^+) from the cell while simultaneously importing potassium ions (K^+) into the cell [4]. By extruding Na^+, it critically regulates cellular osmotic pressure. In addition, the Na^+ gradient established by the pump supports secondary transport processes, such as Na^+/Ca^{2+} exchanger-mediated Ca^{2+} extrusion and Na^+/H^+ exchanger-dependent H^+ removal, which help maintain Ca^{2+} homeostasis and pH equilibrium [4]. Moreover, Na^+/K^+-ATPase is essential for protein synthesis, as it maintains the intracellular K^+ at an appropriate level, a prerequisite for the

formation of the 80S initiation complex and efficient translation elongation [5, 6]. Na^+/K^+-ATPase is composed of a catalytic α-subunit, a regulatory β-subunit, and an auxiliary FXYD protein. Four isoforms of the α-subunit have been identified in humans (ATP1A1-4) [7, 8]. Among them, ATP1A1 is overexpressed in TNBC [9, 10]. Moreover, loss-of-function studies have shown that ATP1A1 knockdown reduced the viability and sphere-forming capacity of TNBC cells, suggesting that ATP1A1 is a potential therapeutic target for TNBC [10].

Hellebrigenol is a bioactive steroid extracted from *Helleborus niger* [11]. A recent study reported that it significantly reduced the viability of hepatocellular carcinoma cells in vitro; however, the anti-TNBC activity of hellebrigenol and its underlying mechanisms remain unclear [12]. This study aimed to evaluate the anti-TNBC effects of hellebrigenol and to determine whether its cytotoxicity is mediated by ATP1A1 inhibition and subsequent disruption of ion homeostasis.

2. Materials and Methods

2.1. Materials

The human TNBC cell lines Hs578T (Cat# CL-0114), SUM159PT (Cat# CL-0622), BT-549 (Cat# CL-0041), and the non-tumorigenic mammary epithelial cell line MCF10A (Cat# CL-0525), together with MCF10A complete medium (Cat# CM-0525) and fetal bovine serum (FBS, Cat# 164210), were obtained from Procell Life Science & Technology Co., Ltd. DMEM (Cat# C11965500BT), RPMI-1640 (Cat# C22400500BT), prestained protein ladder (Cat# 26619), and penicillin/streptomycin (Cat# 15140122) were purchased from Thermo Fisher Scientific. Insulin (Cat# P3376), methionine/serum-free DMEM (Cat# C0891), methionine/serum-free RPMI-1640 (Cat# C0893), Annexin V-FITC Apoptosis Detection Kit (Cat# C1062L), NP-40 (Cat# P0013F), PMSF Solution (Cat# PST507), puromycin dihydrochloride (Cat# Y174907), RIPA Lysis Buffer (Cat# P0013B), and BeyoClick™ HPG Protein Synthesis Assay Kit (Cat# P1215L) were purchased from Beyotime Biotechnology. Sodium chloride-free RPMI-1640 medium (Cat# YC-1064) and Sodium chloride-free DMEM medium (Cat# YC-2112) were purchased from Yuchun Biology Co., Ltd. CCK-8 reagent (Cat# C0005), along with Z-VAD-FMK (Cat# T7020), necrostatin-1 (Cat# T1847), ferrostatin-1 (Cat# T6500), 3-methyladenine (Cat# T1879), and chloroquine (Cat# T8689), were obtained from TargetMol. Hellebrigenol (purity 95%, Cat# PS1585) was acquired from Chengdu Push Bio-Technology Co., Ltd. Crystal Violet Stain Solution (0.5%, Cat# 60506ES60) was purchased from Yeasen Biotechnology Co., Ltd. Sodium chloride (Cat# C111538) and potassium chloride (Cat# P112144) were purchased from Aladdin Biochemical Technology Co., Ltd. ATP1A1 polyclonal antibody (Cat# 14418-1-AP), β-actin monoclonal antibody (Cat# 66009-1-Ig), HRP-conjugated goat anti-rabbit IgG (H + L) (Cat# SA00001-2) and HRP-conjugated goat anti-mouse IgG (H + L) (Cat# SA00001-1) were obtained from Proteintech Group, Inc. Na^+/K^+-ATPase Activity Assay Kit (Cat# AKOP001C) was obtained from Boxbio Co., Ltd. Sodium indicator SBFI AM (Cat# MX4509) and Pluronic® F-127 (Cat# MS4301) were purchased from Maokangbio Co., Ltd.

2.2. Cell culture

Hs578T cells were grown in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. SUM159PT cells were cultivated in RPMI-1640 medium with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. BT549 cells were grown in RPMI-1640 medium with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 10 μg/mL insulin. The human normal breast epithelial cell line MCF10A was cultured in a commercially available complete growth medium recommended for MCF10A cells. Cells were maintained at 37°C in a humid environment with 5% CO_2. All cultures were examined for mycoplasma contamination and used at early passages (< 15). The cell lines were identified by STR profiling.

2.3. CCK-8 assay

Cells were seeded at a density of 4,000 cells/well in 96-well plates, allowed to attach for 24 h and then treated with the indicated compounds for 48 h. The cell viability was determined by adding 10 μL of CCK-8 reagent to each well, incubating for 1 h at 37°C and measuring the absorbance at 450 nm using a microplate reader (Multiskan FC, Thermo Scientific). The inhibitory rate was calculated as (Ac-As)/(Ac-Ab) × 100%, where Ac, As and Ab represent the absorbance of the control, sample, and blank wells, respectively.

2.4. Colony formation assay

Hs578T and SUM159PT cells were seeded in 6-well plates at 400 cells per well and allowed to attach for 48 h. Subsequently, the cultures were incubated in complete medium containing hellebrigenol for 12 days, with the medium replaced every 4 days. After the treatment period, the medium was removed and the wells were washed once with PBS. The colonies were fixed with 4% paraformaldehyde for 10 min at room temperature and stained with 0.5% crystal violet for 20 min in darkness. The excess stain was then washed thoroughly with tap water and the plates were dried before being photographed by using a gel documentation system (ChemiDoc MP, Bio-Rad).

2.5. Cell death assay

Hs578T and SUM159PT cells were seeded in 6-well plates and treated with hellebrigenol at the specified concentrations for 48 h. After treatment, cells were collected by centrifugation at 800 *rpm*, then washed twice with cold PBS and resuspended in 1× binding buffer at a concentration of 1× 10^6 cells/mL. The cells were then stained with Annexin V-FITC and propidium iodide (PI) in the dark for 15 min. Subsequently, the stained cells were analyzed using a flow cytometer (CytoFLEX, Beckman Coulter).

2.6. Molecular docking

The chemical structure of hellebrigenol was obtained from PubChem and converted into PDB format using Open Babel 2.3.2. The crystal structure of ATP1A1 (PDB ID: 7wyt) was downloaded from the Protein Data Bank and processed with PyMOL 2.3.4, in which water molecules and the initial ligand were deleted. Subsequently, hydrogen atoms were added and neutralized using AutoDock Tools. The receptor and the ligand were then converted to PDBQT format and subjected to docking using AutoDock Vina

1.1.2. The binding interactions of the docking results were analyzed by Protein-Ligand Interaction Profiler.

2.7. Cellular thermal stability assay

Hs578T and SUM159PT cells were grown in 10 cm dishes until they reached 80% confluence. After washing the cells with PBS once, they were scraped on ice and pelleted by centrifugation at 800 *rpm*. The pellets were resuspended in 1 mL of PBS containing 0.4% NP-40 and 1 mM PMSF, and sonicated on ice (200 W, 3-s pulses with 10-s intervals, 30 cycles). The lysates were clarified by centrifugation at 8,000 *rpm* for 10 min at 4°C, and the supernatant was divided into two portions for treatment with 0.1% DMSO (as control) or 1 μM hellebrigenol for 30 min on ice. Each sample was then divided into nine equal parts and subjected to a temperature gradient (37–69°C, 3 min) in a thermal cycler (T100, Bio-Rad). After being kept at room temperature for 5 min, the aliquots were put back to ice, mixed with 10 μL of 5× loading buffer, heated at 100°C for 5 min and finally analyzed by Western blotting using an ATP1A1 polyclonal antibody.

2.8. Na^+/K^+-ATPase activity assay

Hs578T and SUM159PT cells were cultivated in 6-well plates until reaching 80% confluence, and then they were exposed to hellebrigenol for 2 h at concentrations of 10–40 nM and 20–80 nM, respectively. After treatment, the cells were harvested and lysed. The Na^+/K^+-ATPase activity was determined by measuring the ATP hydrolysis using the Na^+/K^+-ATPase Assay Kit according to the manufacturer's protocol. The results were normalized to untreated controls, which were set to 100%.

2.9. Establishment of stable ATP1A1(Q111R)-expressing cell lines

A lentiviral vector encoding the rat ATP1A1(Q111R) mutant was generated as previously reported [13]. Hs578T and SUM159PT cells were transduced at a multiplicity of infection (MOI) of 5 and selected with puromycin (2 μg/mL for Hs578T and 1 μg/mL for SUM159PT) for 7–10 days. ATP1A1(Q111R) overexpression was confirmed by Western blotting.

2.10. Intracellular Na^+ measurement

The sodium indicator SBFI AM was dissolved in HBSS to prepare a 20 μM stock solution which was then mixed in a volume ratio of 1:1 (v/v) with 0.02% Pluronic® F-127. Hs578T and SUM159PT cells were seeded in 96-well plates and cultured until they reached 50% confluence, after which they were treated with hellebrigenol at 50 nM (for Hs578T) or 100 nM (for SUM159PT) for 2 h. Subsequently, the cells were washed twice with PBS and incubated with 90 μL of HBSS per well, together with 10 μL of the working solution of SBFI AM and the original concentration of hellebrigenol. The cells were then kept at 37°C in the dark for 1 h. After washing twice with PBS, the cells were observed under a fluorescence microscope (IX73; Olympus) using excitation/emission wavelengths of 340/505 nm and an exposure time of 200 ms.

2.11. Protein synthesis assay

Hs578T and SUM159PT cells were grown in 6-well plates until reaching 80% confluence. After removing the medium, the cells were washed twice with PBS solution and then incubated in 2 mL of methionine/serum-free medium containing 1× L-homopropargylglycine (HPG) working solution and various concentrations of hellebrigenol. After 2 h of treatment, cells were harvested and proteins were extracted. Newly synthesized proteins were detected by click chemistry conjugation using the BeyoClick™ HPG Protein Synthesis Assay Kit according to the manufacturer's instructions.

2.12. Statistical analysis

Data are shown as mean ± standard deviation (SD) from three independent biological replicates. Statistical tests were carried out with GraphPad Prism (version 9.0.0; Dotmatics). The difference between two groups was tested by an unpaired two-tailed Student's t-test, whereas comparisons among three or more groups were performed by one-way ANOVA followed by Dunnett's multiple comparisons test. A P value < 0.05 was considered statistically significant.

3. Results

3.1. Hellebrigenol inhibits proliferation and induces cell death in TNBC cells

CCK-8 assays showed that hellebrigenol (Fig. 1a) effectively suppressed the proliferation of Hs578T, SUM159PT, and BT549 cells, with IC_{50} values of 17.5 nM, 73.1 nM, and 56.8 nM, respectively. Of note, its cytotoxicity in normal mammary epithelial MCF10A cells (IC_{50}: 155.4 nM) was much lower than that in TNBC lines (Fig. 1b). The colony formation assay confirmed that hellebrigenol reduced the colony-forming ability of TNBC cells (Fig. 1c). In terms of cell morphology, hellebrigenol induced distinct alterations: Hs578T cells exhibited apoptotic features, such as cell shrinkage and rounding, whereas SUM159PT cells displayed necrotic or pyroptotic features, including cellular swelling (Fig. 1d). Flow cytometric analysis with Annexin V/PI double staining confirmed that hellebrigenol treatment significantly increased the proportion of Annexin V-positive cells in both cell lines (Fig. 1e). Moreover, both apoptosis and necrosis inhibitors partially rescued Hs578T cells, whereas only the necrosis inhibitor was effective in SUM159PT cells, indicating that the death pathways engaged by hellebrigenol are cell-type-dependent (**Fig S1**).

3.2. Hellebrigenol targets ATP1A1 to induce cytotoxicity in TNBC cells

Molecular docking predicted a strong binding affinity between hellebrigenol and ATP1A1 (ΔG = -10.0 kcal/mol), with the interaction stabilized by hydrophobic contacts (Phe316, Phe783, Phe786, Ala323, and Ile800) and polar interactions (Gln111 and Gln119) (Fig. 2a). This binding was validated by CETSA, which showed that hellebrigenol markedly enhanced the thermal stability of ATP1A1 (Fig. 2b). Given that ATP1A1 is the catalytic subunit of Na^+/K^+-ATPase, we reasoned that hellebrigenol binding would

compromise its ATP hydrolytic activity. Indeed, enzymatic assays confirmed that hellebrigenol significantly suppressed Na^+/K^+-ATPase activity in TNBC cells (Fig. 2c). To functionally validate ATP1A1 as the relevant target, we introduced the ATP1A1(Q111R) mutant, which disrupts the hellebrigenol-binding interface, and found that its expression completely abrogated hellebrigenol-induced cytotoxicity (Fig. 2d, e). Together, these results establish ATP1A1 as the critical molecular target of hellebrigenol and pinpoint Gln111 as the essential binding residue.

3.3. Hellebrigenol exerts cytotoxic effects on TNBC cells by disrupting ion homeostasis

As ATP1A1 mediates Na^+/K^+ exchange via E1-to-E2 conformational transitions [7], we hypothesized that ATP1A1 inhibition by hellebrigenol would disrupt intracellular ion homeostasis. Consistent with this, Na^+ imaging revealed that hellebrigenol treatment induced cytoplasmic Na^+ accumulation in TNBC cells (Fig. 3a). We further found that exogenous Na^+ supplementation potentiated hellebrigenol-induced cytotoxicity (Fig. 3b). Moreover, activation of TRPM4, a sodium influx channel, augmented the cytotoxic effect of hellebrigenol (Fig. 3c, S2). Conversely, reducing extracellular Na^+ concentration attenuated hellebrigenol-mediated cytotoxicity (Fig. 3d). Collectively, these results indicate that the cytotoxicity of hellebrigenol is at least partially attributable to Na^+ overload resulting from ATP1A1 inhibition.

Given that inhibition of ATP1A1 would be expected to deplete intracellular K^+, and K^+ is essential for protein translation, we examined whether hellebrigenol affects protein synthesis in TNBC cells, and indeed found that hellebrigenol markedly suppressed protein synthesis (Fig. 3e) [5, 6]. Moreover, overexpression of the ATP1A1(Q111R) mutant significantly alleviated this translational suppression (Fig. 3f). In parallel, extracellular K^+ supplementation effectively protected cells from hellebrigenol-induced cytotoxicity (Fig. 3g). These findings indicate that K^+ depletion resulting from ATP1A1 inhibition contributes to hellebrigenol-induced cytotoxicity, likely through suppression of protein synthesis.

4. Discussion

This study demonstrates that hellebrigenol can induce cell death in TNBC cells. Mechanistically, hellebrigenol binds to ATP1A1, inhibits the activity of Na^+/K^+-ATPase, and disturbs the intracellular ion balance. Although hellebrigenol exhibited strong cytotoxicity across all three TNBC cell lines (Hs578T, SUM159PT, and BT549), its cytotoxic effect was more pronounced in Hs578T cells (Fig. 1). We speculated that this differential sensitivity may stem from the difference in intracellular Na^+ levels, as Hs578T cells have a higher basal Na^+ content (Fig. 3a), which rendered them more susceptible to Na^+ overload upon ATP1A1 inhibition. Moreover, hellebrigenol caused distinct types of cell death, as both apoptosis and necrosis inhibitors partially rescued the Hs578T cells, whereas only the necrosis inhibitor was effective in SUM159PT cells (**Fig S1**). It should be noted that the overall protection afforded by

these inhibitors was modest, which suggests that other unidentified mechanisms may also contribute to the cytotoxicity of hellebrigenol.

Mechanistic investigation revealed that ATP1A1 is a direct target of hellebrigenol, and that Gln111 of ATP1A1 serves as a critical binding site, as overexpression of the ATP1A1(Q111R) mutant completely reversed hellebrigenol-induced cytotoxicity in TNBC cells (Fig. 2e). Gln111 was previously confirmed as a critical binding residue for ouabain, indicating that hellebrigenol inhibits ATP1A1 via a mechanism similar to that of ouabain [14]. ATP1A1 is a catalytic subunit of Na^+/K^+-ATPase; its inhibition is expected to disrupt intracellular ion homeostasis. Indeed, our study confirmed this (Fig. 3a). “NECSO” (necrosis by sodium overload) has recently been proposed as a novel form of cell death [15]. In line with this notion, we found that reducing extracellular Na^+ concentration attenuated hellebrigenol-induced cytotoxicity, suggesting that hellebrigenol may induce TNBC cell death via NECSO. Intriguingly, activation of TRPM4, a Na^+ channel highly expressed in breast cancer (including TNBC), significantly enhanced the cytotoxic effect of hellebrigenol across multiple TNBC cell lines (Fig. 3d, S2) [16–19]. These results indicate that the concurrent activation of TRPM4 and the inhibition of ATP1A1, both resulting in an elevation in intracellular Na^+, may represent a potential therapeutic strategy for TNBC.

In addition to Na^+ extrusion, Na^+/K^+-ATPase is also involved in K^+ uptake. It is therefore probable that the inhibition of ATP1A1 by hellebrigenol would lead to a depletion of intracellular K^+. Since K^+ is necessary for ribosome function and translation, we reasoned that a drop in K^+ levels would affect protein synthesis [5]. This hypothesis has been confirmed as hellebrigenol significantly reduced the total protein synthesis in TNBC cells (Fig. 3e). Furthermore, adding KCl rescued the cell viability (Fig. 3g), which supports the notion that K^+ depletion contributes to hellebrigenol-induced cytotoxicity. In addition to its function in translation, K^+ has an important role in maintaining the stability of DNA and mRNA, for example, by stabilizing G-quadruplexes, a nucleic acid structure related to chromosome organization and transcription, as well as in regulating chaperone activities, such as Hsc70 [20–23]. It is therefore tempting to speculate that hellebrigenol may exert additional cytotoxic effects by interfering with these K^+-dependent processes.

In recent years, increasing evidence has linked ATP1A1 (or Na^+/K^+-ATPase) to tumor immunity, metastasis, and drug resistance [24–29]. By establishing hellebrigenol as a bona fide ATP1A1 inhibitor, our study provides a foundation for future investigations into its regulatory roles in these processes. Some limitations of our research should be acknowledged. Firstly, although we confirmed ATP1A1 as the functional target, the possibility of off-target effects, especially the interaction with other Na^+/K^+-ATPase α isoforms, cannot be ruled out. Secondly, the present findings about hellebrigenol are based on in vitro studies; the in vivo anti-TNBC efficacy and safety of hellebrigenol warrant further investigation.

In conclusion, this study establishes hellebrigenol as a bona fide ATP1A1 inhibitor with potent anti-TNBC activity, and demonstrates that its cytotoxicity against TNBC cells is mediated through intracellular Na^+ overload and K^+ depletion.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This research was funded by the National Natural Science Foundation of China, grant number: 82004009 and the Science and Technology Plan Program of Guizhou Province, grant number: [2018], 5772-071).

Author Contribution

Conceptualization, Liwen Shen; methodology, Juan Li, Xiaorong Huang and Ying Xu; formal analysis, Liyao Yang; investigation, Juan Li, Xiaorong Huang, Ying Xu and Shuangshuang Fang; resources, Danli Yang; data curation, Liyao Yang; writing—original draft preparation, Yu Wang; writing—review and editing, Liwen Shen; visualization, Liyao Yang; supervision, Danli Yang; project administration, Yu Wang; funding acquisition, Liwen Shen. All authors reviewed the manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/doi/s1,Supplementary_Fig.docx; Supplementary_Raw_WB.docx.

References

1. Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2021;71:209–49. 10.3322/caac.21660.
2. Zagami P, Carey LA. Triple negative breast cancer: Pitfalls and progress. npj Breast Cancer. 2022;8:95. 10.1038/s41523-022-00468-0.
3. Bianchini G, De Angelis C, Licata L, Gianni L. Treatment landscape of triple-negative breast cancer-expanded options, evolving needs. Nat Rev Clin Oncol. 2022;19:91–113. 10.1038/s41571-021-00565-2.

4. Contreras RG, Torres-Carrillo A, Flores-Maldonado C, Shoshani L, Ponce A. Na+/K+-ATPase: More than an electrogenic pump. Int J Mol Sci. 2024;25:6122. 10.3390/ijms25116122.
5. Perne A, Muellner MK, Steinrueck M, Craig-Mueller N, Mayerhofer J, Schwarzinger I, Sloane M, Uras IZ, Hoermann G, Nijman SM, et al. Cardiac glycosides induce cell death in human cells by inhibiting general protein synthesis. PLoS ONE. 2009;4:e8292. 10.1371/journal.pone.0008292.
6. Cerella C, Gajulapalli SR, Lorant A, Gerard D, Muller F, Lee Y, Kim KR, Han BW, Christov C, Récher C, et al. ATP1A1/BCL2L1 predicts the response of myelomonocytic and monocytic acute myeloid leukemia to cardiac glycosides. Leukemia. 2024;38:67–81. 10.1038/s41375-023-02076-8.
7. Moreno C, Yano S, Bezanilla F, Latorre R, Holmgren M. Transient electrical currents mediated by the Na^+/K^+-ATPase: a tour from basic biophysics to human diseases. Biophys J. 2020;119:236–42. 10.1016/j.bpj.2020.06.006.
8. Obradovic M, Sudar-Milovanovic E, Gluvic Z, Banjac K, Rizzo M, Isenovic ER. The Na^+/K^+-ATPase: a potential therapeutic target in cardiometabolic diseases. Front Endocrinol. 2023;14:1150171. 10.3389/fendo.2023.1150171.
9. Kim Y, Ko JY, Kong HK, Lee M, Chung W, Lim S, Son D, Oh S, Park JW, Kim DY, et al. Hypomethylation of ATP1A1 is associated with poor prognosis and cancer progression in triple-negative breast cancer. Cancers. 2024;16:1666. 10.3390/cancers16091666.
10. Bogdanov A, Moiseenko F, Dubina M. Abnormal expression of ATP1A1 and ATP1A2 in breast cancer. *F1000Research* 2017, *6*, 10. 10.12688/f1000research.10481.1
11. Yoshizawa Y, Yokosuka A, Inomata M, Iguchi T, Mimaki Y. Steroidal constituents in the whole plants of *Helleborus niger* and their cytotoxic activity in vitro. Phytochemistry. 2025;29:4272. 10.1016/j.phytochem.2024.114272.
12. Han L, Wang H, Si N, Ren W, Gao B, Li Y, Yang J, Xu M, Zhao H, Bian B. Metabolites profiling of 10 bufadienolides in human liver microsomes and their cytotoxicity variation in HepG2 cell. Anal Bioanal Chem. 2016;408:2485–95. 10.1007/s00216-016-9345-y.
13. Guerrero A, Herranz N, Sun B, Wagner V, Gallage S, Guiho R, Wolter K, Pombo J, Irvine EE, Innes AJ, et al. Cardiac glycosides are broad-spectrum senolytics. Nat Metab. 2019;1:1074–88. 10.1038/s42255-019-0122-z.
14. Price EM, Rice DA, Lingrel JB. Structure-function relationships in the Na,K-ATPase alpha subunit: site-directed mutagenesis of glutamine-111 to arginine and asparagine-122 to aspartic acid generates a ouabain-resistant enzyme. Biochemistry. 1988;27:8400–8. 10.1021/bi00422a016.
15. Fu W, Wang J, Li T, Qiao Y, Zhang Z, Zhang X, He M, Su Y, Zhao Z, Li C, et al. Persistent activation of TRPM4 triggers necrotic cell death characterized by sodium overload. Nat Chem Biol. 2025;21:1238–49. 10.1038/s41589-025-01841-3.
16. Wong KK, Hussain FA. TRPM4 is overexpressed in breast cancer associated with estrogen response and epithelial-mesenchymal transition gene sets. PLoS ONE. 2020;15:e0233884. 10.1371/journal.pone.0233884.

17. Zhu Y, Guo Y, Su Y, Zhang Z, Lu Y, Zhang X, Pang H. Necrosis by sodium overload-associated genes TRPM4 and SLC9A1: biological roles and clinical implications in breast cancer progression. Front Immunol. 2025;16:1623511. 10.3389/fimmu.2025.1623511.
18. Kong Q, Wu Y, Na Z, Yang Q, Xu L. TRPM4: a double-edged sword target driving malignant progression of triple-negative breast cancer and mediating sodium overload-induced cell death. Chin Sci Bull. 2026;71:3253–65. 10.1360/CSB-2025-5614.
19. Reinhold WC, Marangoni E, Elloumi F, Montagne R, Varma S, Wang Y, Rezai K, Morriset L, Dahmani A, El Botty R, et al. Acetalax and Bisacodyl for the treatment of triple-negative breast cancer: a combined molecular and preclinical study. Cancer Res Commun. 2025;5:375–88. 10.1158/2767-9764.CRC-24-0435.
20. Lopina OD, Sidorenko SV, Fedorov DA, Klimanova E. A. G-quadruplexes as sensors of intracellular Na^+/K^+ ratio: potential role in regulation of transcription and translation. Biochem (Moscow). 2022;87:S262–77. 10.1134/S0006297924140153.
21. Robinson J, Raguseo F, Nuccio SP, Liano D, Di Antonio M. DNA G-quadruplex structures: more than simple roadblocks to transcription? Nucleic Acids Res. 2021;49:8419–31. 10.1093/nar/gkab609.
22. Zhang Y, Wang L, Wang F, Chu X, Jiang JH. G-quadruplex mRNAs silencing with inducible ribonuclease targeting chimera for precision tumor therapy. J Am Chem Soc. 2024;146:15815–24. 10.1021/jacs.4c02091.
23. O'Brien MC, McKay DB. How potassium affects the activity of the molecular chaperone Hsc70. I. Potassium is required for optimal ATPase activity. J Biol Chem. 1995;270:2247–50. 10.1074/jbc.270.5.2247.
24. Ren Y, Yu J, Cheng X, Kinghorn AD. Na^+/K^+-ATPase, cardiac glycosides, and tumor immunity. Front Chem. 2026;14:1811060. 10.3389/fchem.2026.1811060.
25. Shandell MA, Capatina AL, Lawrence SM, Brackenbury WJ, Lagos D. Inhibition of the Na^+/K^+-ATPase by cardiac glycosides suppresses expression of the IDO1 immune checkpoint in cancer cells by reducing STAT1 activation. J Biol Chem. 2022;298:101707. 10.1016/j.jbc.2022.101707.
26. Gkountela S, Castro-Giner F, Szczerba BM, Vetter M, Landin J, Scherrer R, Krol I, Scheidmann MC, Beisel C, Stirnimann CU, et al. Circulating tumor cell clustering shapes DNA methylation to enable metastasis seeding. Cell. 2019;176:98–e11214. 10.1016/j.cell.2018.11.046.
27. Kurzeder C, Nguyen-Sträuli BD, Krol I, Ring A, Castro-Giner F, Nüesch M, Asawa S, Zhang YW, Budinjas S, Gvozdenovic A, et al. Digoxin for reduction of circulating tumor cell cluster size in metastatic breast cancer: a proof-of-concept trial. Nat Med. 2025;31:1120–4. 10.1038/s41591-024-03486-6.
28. Liu D, Liu M, Lv D, Li Y, Guo H, Lu B, Leng H, Yan R, Yu H, Blom T, et al. LAPTM4B confers resistance to EGFR-TKIs by suppressing the proteasomal degradation of ATP1A1 in non-small cell lung cancer. Int J Biol Sci. 2026;22:1–24. 10.7150/ijbs.115365.
29. Hu J, Meng F, Lv L, Hong F, He Q, Zhu Q, Tian T, Chang N, Zhang S, Yi Q, et al. GPR37-enhanced ubiquitination of ATP1A1 inhibits tumor progression and radiation resistance in esophageal

squamous cell carcinoma. Cell Death Dis. 2024;15:933. 10.1038/s41419-024-07240-1.

Figures

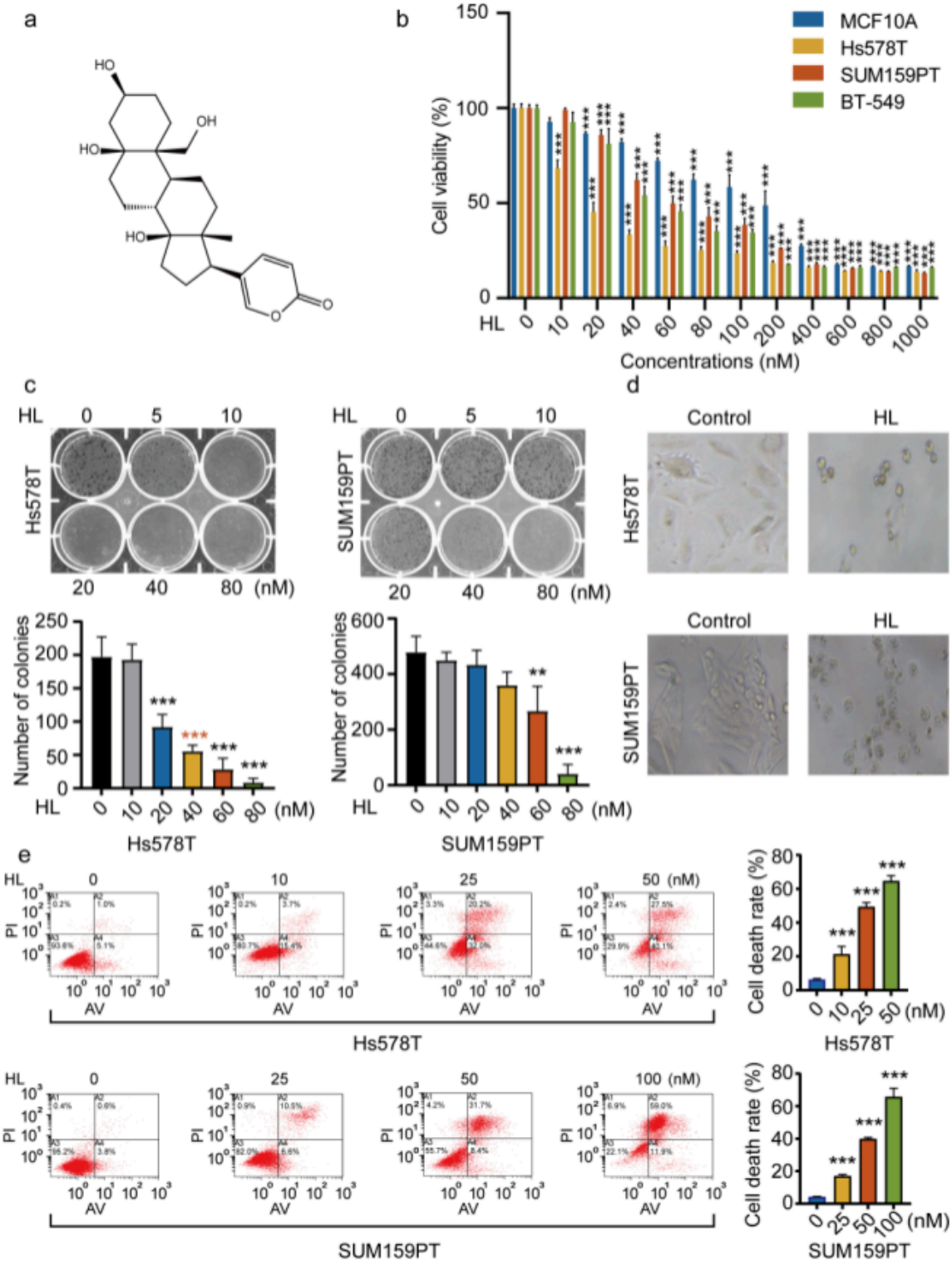


Figure 1

Hellebrigenol inhibits proliferation and induces cell death in triple-negative breast cancer (TNBC) cell lines. (**a**) Chemical structure of hellebrigenol. (**b**) Cytotoxic effects of hellebrigenol on Hs578T, SUM159PT, BT-549, and MCF10A cells after 48 h of treatment. (**c**) Clonogenic ability of TNBC cells treated with hellebrigenol for 12 days. (**d**) Morphological changes in TNBC cells upon hellebrigenol treatment (Hs578T: 100 nM, 48 h; SUM159PT: 200 nM, 48 h). (**e**) Cell death induction by hellebrigenol in TNBC cells after 48 h of treatment. Data are presented as mean ± SD from three independent experiments (n = 3). *P < 0.05, **P < 0.01, ***P< 0.001 vs. control. HL, hellebrigenol; AV, Annexin V-FITC; PI, propidium iodide.

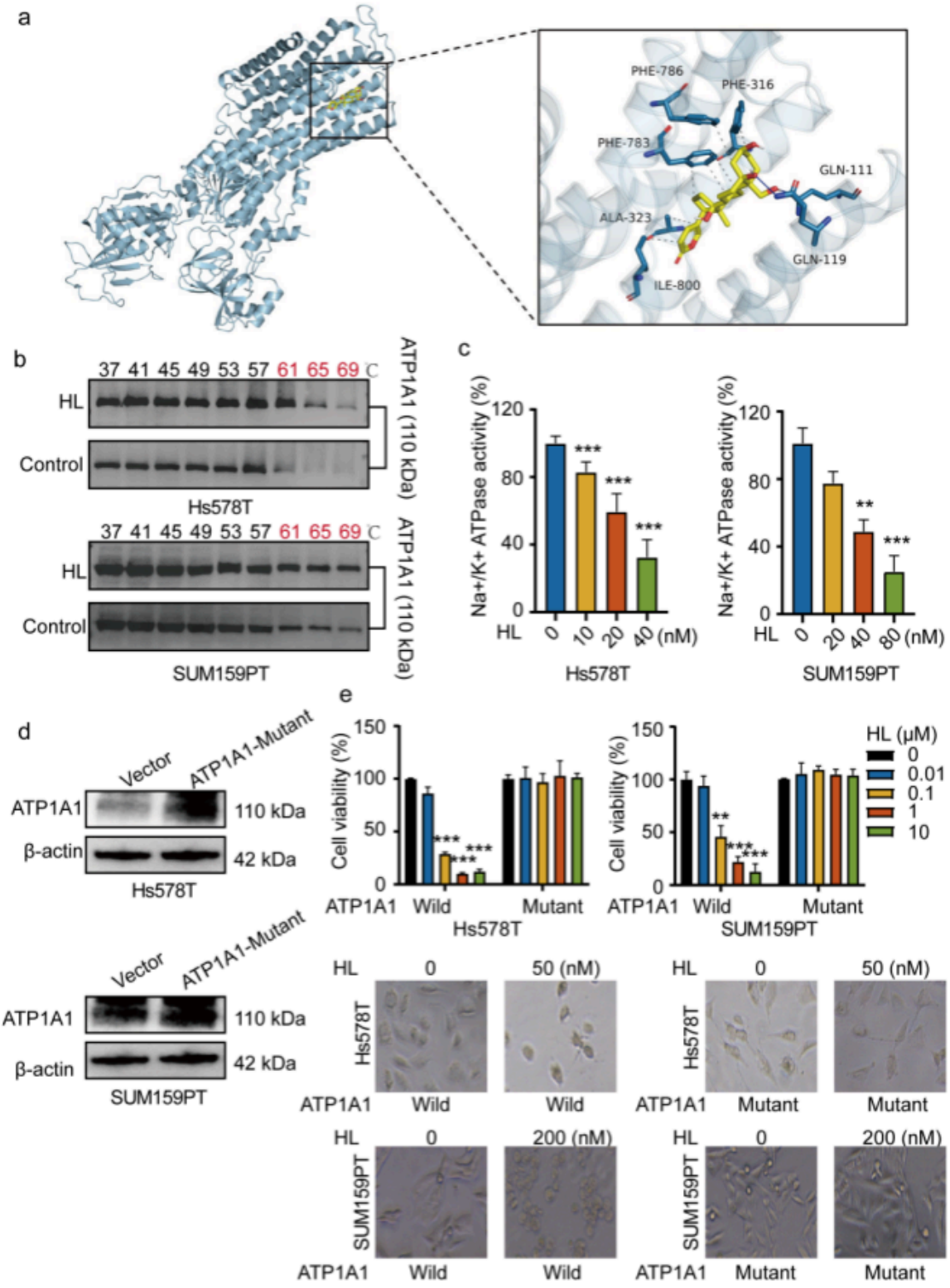


Figure 2

Hellebrigenol targets ATP1A1 to induce cytotoxicity in TNBC cells. (**a**) Predicted binding mode of hellebrigenol to ATP1A1. (**b**) Thermal stability of ATP1A1 with or without hellebrigenol (1 μM). (**c**) Na^+/K^+-ATPase activity in TNBC cells treated with hellebrigenol. (**d**) Western blot confirming ATP1A1(Q111R) overexpression. (**e**) The Q111R mutation abrogates hellebrigenol-ATP1A1 binding and reverses hellebrigenol-induced cytotoxicity. Top: viability of wild-type and

ATP1A1(Q111R)-overexpressing TNBC cells after 48 h of hellebrigenol treatment. Bottom: morphological changes in wild-type and ATP1A1(Q111R)-overexpressing TNBC cells after 48 h of hellebrigenol treatment. Data are presented as mean ± SD from three independent experiments (n = 3). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. control. HL, hellebrigenol.

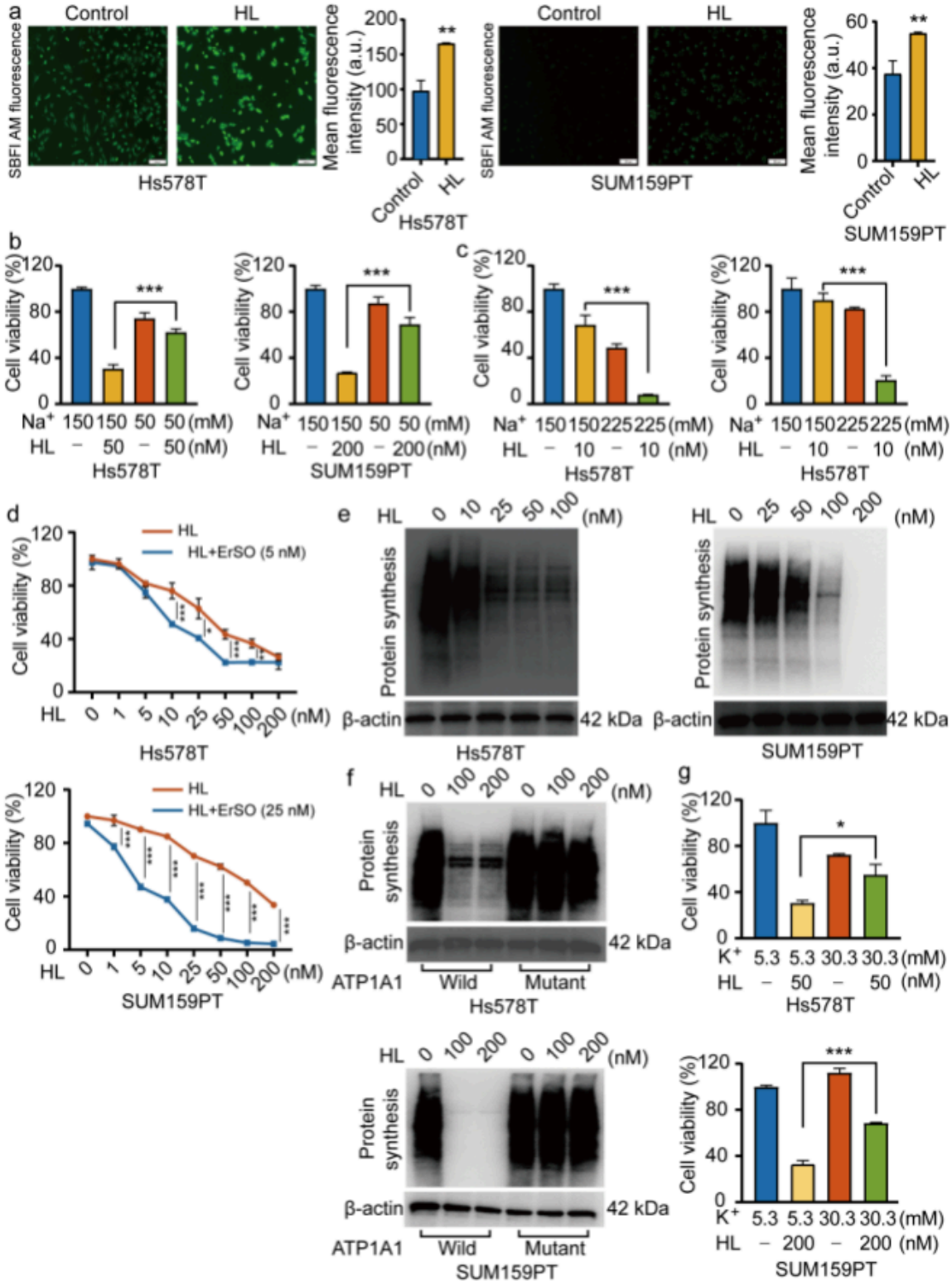


Figure 3

Hellebrigenol induces cytotoxicity in TNBC cells through ATP1A1 inhibition-mediated ion perturbation. (**a**) Intracellular Na^+ levels in TNBC cells treated with hellebrigenol for 2 h (Hs578T: 50 nM; SUM159PT: 100 nM). (**b**) Effects of extracellular Na^+ withdrawal (low Na^+: 50 mM) on hellebrigenol-induced cytotoxicity after 48 h (normal Na^+: 150 mM). (**c**) Effects of Na^+ supplementation (high Na^+: 225 mM) on hellebrigenol-induced cytotoxicity after 48 h (normal Na^+: 150 mM). (**d**) Cytotoxic effects of hellebrigenol in the presence or absence of ErSO after 48 h. (**e**) Protein synthesis levels in TNBC cells treated with hellebrigenol for 6 h. (**f**) Protein synthesis levels in wild-type and ATP1A1(Q111R)-overexpressing TNBC cells after 6 h of hellebrigenol treatment. (**g**) Effects of K^+ supplementation (high K^+: 30.3 mM) on hellebrigenol-induced cytotoxicity after 48 h (normal K^+: 5.3 mM). Data are shown as mean ± SD from three independent experiments (n = 3). Statistical comparisons: (**a**) hellebrigenol-treated groups vs. respective controls; (**b**, **c**, **g**) cell viability compared between normal and altered ion conditions at the same hellebrigenol concentration; (**d**) groups with vs. without ErSO at the same hellebrigenol concentration. *P < 0.05, **P < 0.01, ***P < 0.001 versus respective control (**a**) or as indicated (**b**, **c**, **d**, **f**). HL, hellebrigenol; ErSO, TRPM4 agonist.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigures.docx
- SupplementaryRawWB.docx